Vitamin D Deficiency and Determination of 25-Hydroxyvitamin D by LC-MS/MS

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Vitamin D

- Metabolism and biological actions
- Recommended blood levels
- Ergocalciferol (D₂) vs. cholecalciferol (D₃)
- Methods for measuring vitamin D
- 25OHD by LC-MS/MS
  - Extraction and isotopic dilution
  - Chromatographic separation
  - Detection by mass spectrometer
  - Data reduction and reporting

Nichols Institute, Chantilly, VA
Is Vitamin D a Vitamin?

A compound is called a vitamin when it cannot be synthesized by an organism and must be obtained from the diet.

**Vitamin D is not a vitamin**

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**Biological Actions of Vitamin D**

- **Classical**
  - Intestine: stimulate calcium absorption
  - Bone: bone formation and calcium mobilization
  - Kidney: mediate renal tubular phosphate and calcium reabsorption

- **Non-classical**
  - Dozens of target tissues
  - Anti-proliferative activity
  - Cell differentiation activity
  - Immune system modulation

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Calcium Homeostasis

- Increases excretion of phosphate

Message # 1

Hormone: a complex chemical substance produced in one part or organ of the body that initiates or regulates the activity of an organ or a group of cells in another part of the body.

Vitamin D is a hormone!

Levels of Vitamin D Metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D</td>
<td>0.2 – 20.0</td>
</tr>
<tr>
<td>25OHD</td>
<td>10 – 100</td>
</tr>
<tr>
<td>24,25(OH)₂D</td>
<td>2 – 5</td>
</tr>
<tr>
<td>1,25(OH)₂D</td>
<td>0.015 – 0.060</td>
</tr>
</tbody>
</table>

- ~ 40 minor metabolites of vitamin D
- 25OHD: best indicator of vitamin D status

What is a “healthy” 25OHD level?
What Is Recommended Serum 25OHD Level?

<table>
<thead>
<tr>
<th></th>
<th>ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>From population-based study</td>
<td>9 – 54</td>
</tr>
<tr>
<td>Current reference range</td>
<td>30 – 100</td>
</tr>
<tr>
<td>Recent literature</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Vitamin D council</td>
<td>35 – 55</td>
</tr>
<tr>
<td>IOM</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

Vitamin D varies greatly depending on both season and latitude (sunlight).

Vitamin D Daily Results Distribution

[Graph showing distribution of Vitamin D levels]

Data from Quest Diagnostics Nichols Institute

PTH Suppression Test

[Graph showing PTH suppression test results]

**25OHD – What is “normal”?**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>25OHD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH suppression</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Bone mineral density</td>
<td>36-40</td>
</tr>
<tr>
<td>Fracture prevention</td>
<td>36-40</td>
</tr>
<tr>
<td>Leg function</td>
<td>36-40</td>
</tr>
<tr>
<td>Periodontal disease</td>
<td>36-40</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>36-40</td>
</tr>
</tbody>
</table>

These data suggest that the physiological lower limit of normal for 25OHD is ~35 ng/mL. The upper limit has not been determined but it is probably >100 ng/mL.

**Messages #2**

~45% of U.S. population suffer from vitamin D deficiency and/or insufficiency

**Causes of Vitamin D Deficiency**

Vitamin D deficiency is a disease of the industrial revolution (air pollution) and the use of clothes and sunscreen.
Vitamin D deficiency is the second most common endocrine disorder in this country – next to thyroid disorder.

Diseases Associated with Vitamin D Deficiency in Humans
- Osteoporosis and rickets
- Infectious disease
- Autoimmune diseases
  - Type 1 diabetes
  - Rheumatoid arthritis
  - Multiple sclerosis
- Cancers
  - Breast
  - Colon
  - Ovarian
  - Pancreas
  - Prostate
- Hypertension, cardiovascular disease

Cumulative Probability of 1st CV Event

Participants with hypertension
- 25 OHD <15 ng/mL
- 25 OHD ≥15 ng/mL

Participants w/o hypertension
- 25 OHD <15 ng/mL
- 25 OHD ≥15 ng/mL

Vitamin D Test Volume (2000-2008)

Human Sources of Vitamin D

<table>
<thead>
<tr>
<th></th>
<th>Cholecalciferol (Vitamin D₃)</th>
<th>Ergocalciferol (Vitamin D₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunlight (IU/d)</td>
<td>2,000 – 20,000</td>
<td></td>
</tr>
<tr>
<td>Diet (IU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamins</td>
<td>200 – 1,000</td>
<td>200 – 1,000</td>
</tr>
<tr>
<td>Milk</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>200 - 400</td>
<td></td>
</tr>
<tr>
<td>Drisdol®</td>
<td>50,000</td>
<td></td>
</tr>
<tr>
<td>Mushrooms</td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>

*100 IU/day of vitamin D₃ = 25OHD increase of 1 ng/mL
†1 international unit (IU) = 65 pmol = 25 ng.

Treatment of Vitamin D Deficiency

Drisdol® Tablets
- Only high-dose, prescription form of Vit D in U.S.
- 50,000 IU (1.25 mg) of ergocalciferol (Vit D₂)

U.S. promotes the pharmacological use of ergocalciferol (Vitamin D₂)
Elsewhere, high-dose forms of vitamin D are formulated with cholecalciferol (Vitamin D₃)
## How Much?

### Recommended dietary allowances (RDAs)

<table>
<thead>
<tr>
<th>Age</th>
<th>RDA (IU)</th>
<th>Upper Limit (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 months</td>
<td>400</td>
<td>1,000</td>
</tr>
<tr>
<td>6-12 months</td>
<td>400</td>
<td>1,500</td>
</tr>
<tr>
<td>1-3 years</td>
<td>600</td>
<td>2,500</td>
</tr>
<tr>
<td>4-8 years</td>
<td>600</td>
<td>3,000</td>
</tr>
<tr>
<td>9-70 years</td>
<td>600</td>
<td>4,000</td>
</tr>
<tr>
<td>&gt;70 years</td>
<td>800</td>
<td>4,000</td>
</tr>
</tbody>
</table>

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**How Much?**

- Meta-analysis of randomized control trials (>80,000 fractures total)
- No reduction in fractures when vitamin D dose <400 IU/d
- Higher dose (482-770 IU/d) vitamin D reduced non-vertebral and hip fractures by more than 15%


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**How Much?**

Visit [www.grassrootshealth.com](http://www.grassrootshealth.com), 05/10/2010.
Levels of 25OHD Associated with Disease Prevention

Disease Incidence Prevention by Serum 25(OH)D Level

<table>
<thead>
<tr>
<th>Disease Incidence</th>
<th>Serum 25(OH)D Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 30 ng/mL</td>
</tr>
<tr>
<td></td>
<td>30-50 ng/mL</td>
</tr>
<tr>
<td></td>
<td>&gt; 50 ng/mL</td>
</tr>
</tbody>
</table>

www.grassrootshealth.com, 07/26/2008

Cholecalciferol and Ergocalciferol

Humans make vitamin D3 in the skin
Plants and yeast make vitamin D2

25-Hydroxyvitamin D3
25-Hydroxycholecalciferol
25OHD3

25-Hydroxyvitamin D2
25-Hydroxyergocalciferol
25OHD2

400 daltons
412 daltons

Messages #4

D2 > D3

D2 = D3

or

D2 < D3
LC/MS/MS Data on 25(OH)D vs Plateau Phase of iPTH Suppression

Why Measure Both D₂ and D₃?

56 year-old female at risk for osteoporosis

<table>
<thead>
<tr>
<th></th>
<th>Baseline ng/mL</th>
<th>Post 12 wk Drisdol³ ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD, Total</td>
<td>43</td>
<td>46</td>
</tr>
</tbody>
</table>

³ 50,000 units once weekly.

Why?

- Lab error
- Patient non-compliance
- Seasonal change
- Doctor ordered wrong test
- All of above
Why Measure Both D$_2$ and D$_3$?

<table>
<thead>
<tr>
<th></th>
<th>Baseline ng/mL</th>
<th>Post 12 wk Drisdol$^a$ ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD, Total</td>
<td>43</td>
<td>46</td>
</tr>
<tr>
<td>25OHD$_2$</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>25OHD$_3$</td>
<td>16</td>
<td>36</td>
</tr>
</tbody>
</table>

$^a$ 50,000 units once weekly.

25OHD LC-MS/MS Method

- 2 Simultaneous assays
  - Assay #1: 25OHD$_2$
  - Assay #2: 25OHD$_3$

- Procedure
  - Extraction and isotope dilution via protein precipitation
  - HPLC (high performance liquid chromatography)
  - MS/MS (tandem mass spectrometry)
  - Data reduction and reporting

25OHD (RIA) Step by Step
25OHD (LC-MS/MS) Step by Step

LC-MS/MS System

- Liquid chromatography, tandem mass spectrometry (LC-MS/MS)
- Throughput: ~1,000 cases/d

Chromatographic Separation
Basics of Mass Spectrometry

MS/MS

Sample Preparation

- LC often obviates need for derivatization
- LC compatible with on-line extraction
- LC compatible with direct injection
Ionization Mechanisms

- Electrospray Ionization
- Atmospheric Pressure Chemical Ionization

2002 Nobel prize

"for their development of soft desorption ionization methods for mass spectrometric analyses of biological macromolecules"

John Fenn
Koichi Tanaka

Electrospray Ionization

Drying Gas

ESI needle

To mass spec

Capillary inlet

Mass Analyzers

- Linear quadrupole
- Ion traps
- Triple quadrupole
- Linear quadrupole ion traps
- Time of flight
- QTOF
- Orbitrap
Quadrupole Mass Analyzer

![Image of Quadrupole Mass Analyzer]

2006 Instrument Manual; courtesy of Thermo Electron Corporation

Animation of ion movement because of IE

MRM (Multiple Reaction Monitoring)

<table>
<thead>
<tr>
<th>CE (V)</th>
<th>Q1</th>
<th>Q3</th>
<th>Dwell time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>20</td>
<td>80</td>
<td>100ms</td>
</tr>
<tr>
<td>1500</td>
<td>24</td>
<td>74</td>
<td>200ms</td>
</tr>
<tr>
<td>2000</td>
<td>16</td>
<td>80</td>
<td>200ms</td>
</tr>
<tr>
<td>300</td>
<td>25</td>
<td>74</td>
<td>200ms</td>
</tr>
<tr>
<td>1500</td>
<td>24</td>
<td>74</td>
<td>200ms</td>
</tr>
<tr>
<td>2000</td>
<td>46</td>
<td>80</td>
<td>200ms</td>
</tr>
<tr>
<td>2000</td>
<td>46</td>
<td>80</td>
<td>200ms</td>
</tr>
<tr>
<td>2000</td>
<td>46</td>
<td>80</td>
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</tr>
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<td>46</td>
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<td>200ms</td>
</tr>
<tr>
<td>2000</td>
<td>46</td>
<td>80</td>
<td>200ms</td>
</tr>
</tbody>
</table>
Tandem Mass Spectrometry

- Triple quadrupole mass spectrometer
- 3 quadrupoles
- Q1 = mass filter (for precursor ions)
- Q2 = collision cell
- Q3 = mass filter (for product ions)

2006 Instrument Manual; courtesy of Thermo Electron Corporation

Tandem MS - Cutaway View

2006 Instrument Manual; courtesy of Thermo Electron Corporation

Triple Quadrupole
LC-MS/MS Chromatographs

Blank
0 ng/mL 25OHD₂
0 ng/mL 25OHD₃

High Standard
128 ng/mL 25OHD₂
128 ng/mL 25OHD₃

Data from Quest Diagnostics Nichols Institute

LC-MS/MS Chromatographs

“Normal” patient
35 ng/mL 25OHD₃
<4 ng/mL 25OHD₂

Patient taking vitamin D₂
<4 ng/mL 25OHD₂
85 ng/mL 25OHD₃

Data from Quest Diagnostics Nichols Institute

LC-MS/MS Standard Curves

6-point standard curve: (128, 64, 32, 16, 8, 4 ng/mL)

Data from Quest Diagnostics Nichols Institute
25OHD LC-MS/MS Validation

Reproducibility (%)

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>(20-25 ng/mL)</td>
<td>(45-55 ng/mL)</td>
<td>(95-105 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25OHD₂</td>
<td>9.0</td>
<td>6.9</td>
<td>8.1</td>
</tr>
<tr>
<td>25OHD₃</td>
<td>11.2</td>
<td>7.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Interassay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25OHD₂</td>
<td>9.7</td>
<td>11.6</td>
<td>8.8</td>
</tr>
<tr>
<td>25OHD₃</td>
<td>13.5</td>
<td>10.7</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Data from Quest Diagnostics Nichols Institute

25OHD LC-MS/MS Validation

Sensitivity (limit of quantitation)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass (Da)</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD₂</td>
<td>412</td>
<td>100%</td>
</tr>
<tr>
<td>25OHD₃</td>
<td>400</td>
<td>ND</td>
</tr>
<tr>
<td>6D-25OHD₃</td>
<td>406</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin D₂</td>
<td>396</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>384</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25(OH)₂D₂</td>
<td>428</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25(OH)₂D₃</td>
<td>416</td>
<td>ND</td>
</tr>
<tr>
<td>25,26(OH)₂D₃</td>
<td>416</td>
<td>ND</td>
</tr>
<tr>
<td>1α(OH)D₂</td>
<td>412</td>
<td>ND</td>
</tr>
<tr>
<td>1α(OH)D₃</td>
<td>400</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data from Quest Diagnostics Nichols Institute

25OHD LC-MS/MS Validation

Specificity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass (Da)</th>
<th>Cross-reactivity (25OHD₂)</th>
<th>Cross-reactivity (25OHD₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD₂</td>
<td>412</td>
<td>100%</td>
<td>ND</td>
</tr>
<tr>
<td>25OHD₃</td>
<td>400</td>
<td>ND</td>
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<td>25,26(OH)₂D₃</td>
<td>416</td>
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<td>412</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1α(OH)D₃</td>
<td>400</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data from Quest Diagnostics Nichols Institute

ND: not detected
LC-MS/MS vs. V#4

Figure 1: Scatter plot showing the relative difference between LC-MS/MS and V#4.


LC-MS/MS vs. IAs

Figure 2: Scatter plot with linear fit.

LC-MS/MS Advantages

- Automation – low hands-on-time
- Cost reduction – little reagent cost
- Multiplex – excellent efficiency
- Reproducibility – excellent precision/low CV
- Sensitivity – better than many of the IAs
- Small sample volume – few QNS
- Specificity – no immuno cross reactivity
- Versatility – large and small molecules
- Wide dynamic range – few repeats
### Summary

- Vitamin D deficiency (<20 or <30 ng/mL total 25OHD) is very common
- Two different forms of 25OHD (D2 and D3) – both need to be measured to obtain total 25OHD concentration
- Many causes for vitamin D deficiency
- 25OHD (instead of 1,25 DHVD) is the marker for measuring vitamin D storage status
- Assay standardization is essential (population-based reference ranges are not recommended)

### Summary

- Many implications for 25OHD deficiency
  - Conventional
  - Non-conventional
- Possible indicator/use as a chemo-preventive agent for various diseases/cancers
- Methods for vitamin D determination
- LC-MS/MS should be the method of choice